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Synthesis of Bacterial Flagella

II. PBS1 Transduction of Flagella-specific Markers in *Bacillus subtilis*

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The linkage relationship of mutants involved in the synthesis of flagella was determined by PBS1 transduction. Mutants that affect the structure of flagellin (*hag*) and temperature-sensitive mutants (*flaTS*) that produce flagella when grown at 37 C but not when grown at 46 C were examined. All of the mutants were found to be linked to the *hisA1* marker. The *flaTS* mutants fell into three clusters. Group A contained the majority of mutants which were loosely grouped around the *hag* locus. Group B mutants were segregated from the *hag* locus and appeared closely linked to the phage adsorption site gene (*gtaA*), and group C was only loosely linked to *hisA1* and thus far contains only one mutant. A flagella locus (*ifm*) affecting both the degree of motility and level of flagellation was shown to map near group A. Mutants affecting motility (*mot*) were not linked to *hisA1* by PBS1 transduction. Several markers previously shown to link to *hisA1* were ordered with respect to *hisA1* and the flagellar genes.

Previous work on the genetic control of flagellation in *Bacillus subtilis* (7) has defined the existence of at least three classes of mutation analogous to those found in *Salmonella typhimurium*, *hag*, *fla*, and *mot*. The *hag* group involves modification of the structure of the flagellar filament subunit protein. The wild-type W23 strain, for example, possesses immunologically distinct flagellin (*hag-2*) which differs from wild-type 168 (*hag-1*) in both amino acid composition and peptide sequence (S. Emerson, *personal communication*).

The *fla* mutants phenotypically lack flagella and are presumably defective in functions involved in the synthesis and assembly of the organelle. They are readily isolated in *B. subtilis* but are difficult to examine genetically. The problem stems from the fact that both transduction with SP10 and transformation establish linkage relationships over only relatively short, well-defined intervals of the map, whereas transduction with PBS1, which allows the transfer of extensive fractions of the genome, is mediated by a flagella-specific virus which does not adsorb to *fla* recipient cells (5). To establish a system for both genetic and biochemical study of flagella synthesis, we used the known variants of the structural gene (*hag-1*, *hag-2*, *hag-3*) and also isolated a number of temperature-sensitive mutants (*flaTS*). The *flaTS* mutants allow the adsorption of PBS1 and hence transduction at 37 C, but they do not possess flagella at 46 C and can, therefore, be scored for recombination.

A preliminary report on the mapping of flagella mutants in *B. subtilis* was presented at the 68th Annual Meeting of the American Society for Microbiology, Detroit, Mich., 5-10 May 1968.

MATERIALS AND METHODS

Media. Basal medium was a minimal salts medium (2) supplemented with either 0.1% Casamino Acids and 30 μg of appropriate growth requirements per ml, or, when selective medium was required, 20 μg of all amino acids and requirements per ml excepting the particular growth factor used as a selective agent.

Soft motility agar was composed of basal medium supplemented with 0.4% agar and 0.8% gelatin; when appropriate, sufficient flagella-specific antiserum was added to inhibit motility.

Antibodies and antigens. *B. subtilis* flagellar protein was purified and antisera were prepared as previously described (6).

Nomenclature. To assign consistent designations to flagellar mutations in this study, we have adhered to the conventions proposed by Demerec et al. (3). Since we assume, on the basis of both our data and the data presented by Frankel and Joys (5) that the flagellar antigens represent alternate alleles of a single *hag* gene, the wild-type 168 antigen has been designated *hag-1*; the W23 antigen, *hag-2*; and the straight filament mutation reported by Martinez et al. (8), *hag-3*. Genetic analysis on *B. subtilis* is not refined enough, at this stage, to completely rule out the possibility of multiple *hag* cistrons.

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Strains. Tables 1 and 2 show the properties and origin of strains of *B. subtilis* used and prepared in this investigation. All parent strains were selected for rapid motility by passage through motility tubes.

The selection of *flaTS* mutants may be accomplished in a number of ways. The mutants described in this paper were isolated following treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 100 μg/ml (approximately 50% survival) according to the procedure of Adelberg et al. (1). They were selected by

TABLE 1. *Strains of B. subtilis*

Strain	Origin	Genotype			Derivation
		Auxotrophic markers	*hag*	Other markers	
W23	Spizizen	—	2	—	Wild type
MH-1	Sueoka	*trp-2 lys*	1	*rou-1*	—
BD71	Dubnau	*hisA1, ura, argC4*	1	—	—
BR19	Reilly	*hisA1, trp-2*	1	*rou-1*	—
Rog 1	Rogolsky	—	1	*spoC1*	Marburg wild type cured for sporulation marker with acridine orange
Rog 3	Rogolsky	*hisA1, trp-2*	1	*spoC1*	BR19 transformed with Rog 1 DNA
BR19 (*hag-2*)	—	*hisA1, trp-2*	2	*rou-1*	BR19 transformed with W23 DNA
BR13	Reilly	*ura-1, trp-2*	1	*rou-1*	—
BR13 (*hag-2*)	—	*ura-1, trp-2*	2	*rou-1*	BR13 transformed with W23 DNA
BR85	Reilly	*argC4, trp-2*	1	*rou-1*	—
SB-3	Nester	*hisA1, trp-2, cysB*	1	—	—
FY'A'	Young	*trp-2*	1	*gtaA rou-1*	—
JH1057	Hoch	*trp-2 met-4*	1	*uvr-1*	—
G-2	—	*hisA1, ura, argC4*	2	—	BD71 transformed with W23 DNA
SO-49	Joys (7)	*trp-2*	1	*mot*	—
G-5	—	*hisA1, argC4*	2	*gtaA*	G2 transformed with excess FY'A' DNA selection of *gtaA* by congression eliminating *ura*
G-10	—	*argC4*	2	*gtaA*	G-5 transduced with PBS1 lysate of MH-1
SC-3 and SC-4	Martinez	*trp-2*	3	—	Nonmotile mutant possessing straight flagella; antigenically *hag-1*
G-22	—	*hisA1, ura*	3	—	BD71 transformed with SC-4 DNA
flaTS −1 to *−10* + *flaTS −32* to *−47*+	—	*trp-2, lys*	1	—	Nitrosoguanidine mutagenesis of MH-1
flaTS −18 to *−23*	—	*trp-2, ura*	1	—	Nitrosoguanidine mutagenesis of BR13
flaTS −51	—	*hisA1, ura, argC4*	1	—	Nitrosoguanidine mutagenesis of BD71
G-25	—	*hisA1*	2	*gtaA, uvr-1, ifm-1*	G5 transformed with excess JH1057 DNA
G31	—	*hisA1*	1	*gtaA, uvr-1 ifm-1*	G25 transformed with FY'A' DNA
G26		*hisA1, ura*	2	*uvr-1, ifm-1*	G2 transformed with excess JH1057 DNA

cycling cells through high (46 C) and low (37 C) temperatures and transferring the fraction of the culture which agglutinated at 37 C but not at 46 C with flagella-specific antibodies. The process was repeated four or five times, and the cells were plated in soft motility agar for single colonies at 46 C. Nonmotile clones were picked, restreaked, and tested for their ability to produce flagella at both temperatures. The mutants used for mapping produce few or no flagella at 46 C but do produce flagella at 37 C. A single mutant from each selection run was used.

TABLE 2. *Relative position of hag replacement locus on B. subtilis chromosome*[a]

Recipient strain	Selected auxotrophic markers	Migration thru minimal agar containing antibody	Binding of ^{125}I-*hag-2* antibody[b]
BR-27	*ade-4*	−	<1,000
BR-5	*ade-1*	−	<1,000
BR-62	*ade-5*	−	<1,000
BR-77	*thr-1*	−	<1,000
SB-8	*cysB*	(+)	13,215
BR-19	*hisA1*	+++[c]	25,276[c]
BR-85	*argC4*	−	<1,000
BR-51	*metA7*	−	<1,000
BR-13	*ura-1*	−	<1,000
BR-123	*argO1*	−	<1,000
BR-44	*leu-6*	−	<1,000
BR-84	*phe-3*	−	<1,000
BR-76	*lys-3*	−	<1,000
BR-76	*trp-2*	−	<1,000
BR-50	*met-6*	−	<1,000

[a] Recipient strains (168M auxotrophs, *trp-2*, *hag-1*, X$^-$) were transduced with a lysate of a 168 strain possessing *hag-2* flagella. All recipient strains were obtained from B. Reilly.

[b] Transduced cells were grown overnight in minimal medium and tested for binding of *hag-2* specific antibody. Results are expressed as counts per minute per 10^8 bacteria.

[c] When a transducing lysate of W23 was used, no linkage of the *hag* locus was obtained.

In the course of this work, another flagellar mutant, *ifm*, was found. This mutation leads to an increase both in the motility of the bacteria and in the quantity of flagella per cell. Figure 1 shows that in very soft agar (0.20%) the *ifm* clones spread slightly faster than wild type (*ifm*$^+$). The difference was more clearly observed when 0.4% agar plates were used (Fig. 1). These mutants were selected by picking the leading edge of a wild-type colony grown on 0.4% agar plates. The cells were streaked, and single colonies were picked and tested for increased flagellar antigen and motility.

Other markers used in this study were as follows:

(i) Sporulation mutants, *spoC1* (10), isolated by acridine orange treatment of germinating spores.

(ii) The phage-resistant markers isolated by Young (12; Bacteriol. Proc., p. 56, 1968) which (*gtaA*, *B*, and *C*) affect the glucosylation of teichoic acids in *B. subtilis* cell walls.

(iii) A marker (*uvr-1*) that increases the sensitivity of the cells to ultraviolet light and mitomycin C but does not affect recombination (J. Hoch and C. Anagnostopoulos, *manuscript in preparation*). Recombinants were scored on plates containing mitomycin C (0.05 μg/ml).

(iv) A morphological marker *rou-1* which affects the colony appearance. Wild-type *B. subtilis* strains grown overnight on AK sporulation agar produce rough, pellicle-like colonies, whereas the *rou-1* mutants grow as smooth, soft colonies. The mutant phenotype is found in most 168 M (*trp-2*) derivatives, and can be obtained by treatment of wild-type strains with acridine orange or by growth at 46 C (G. Grant, *unpublished data*).

Bacteriophages PBS1 and Φ25 were obtained from B. Reilly (Scripps Clinic and Research Foundation).

Assay for flagella. Flagella protein was assayed by the procedure of Grant and Simon (6) with the use of purified flagellar-specific ^{125}I-labeled antibodies.

Transduction. PBS1 (11) was used as donor virus throughout. Plaque assays were carried out with *B licheniformis* (ATCC 8480) as the indicator strain

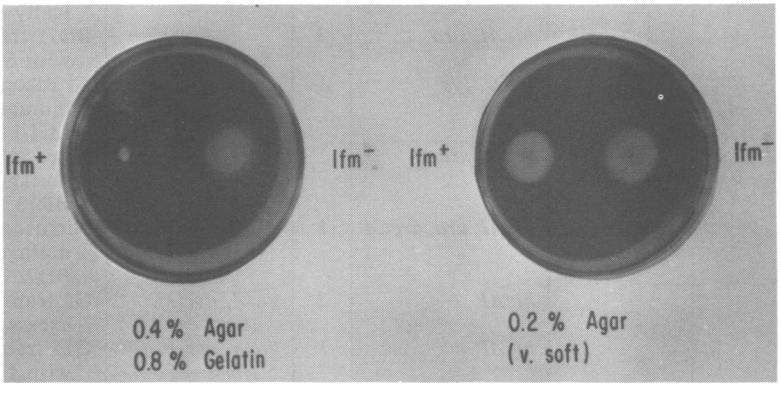

FIG. 1. *Differentiation of ifm alleles by the use of motility agar containing 0.4% agar and 0.8% gelatin or 0.2% agar.*

Stock virus was prepared by infection of an early log culture of *B. licheniformis* in Penassay Broth (Difco). After lysis, the phage were purified and concentrated by differential centrifugation. The preparation of transducing lysates and the transduction were carried out according to the procedures of Reilly and Spizizen (*personal communication*). Donor strains were inoculated into Penassay Broth from overnight Tryptose Blood Agar Base (TBAB; Difco) plates and were grown to an optical density of approximately 150 Klett units; they were then infected with stock PBS1 at a multiplicity of 5. The infected lysate was incubated with shaking for 3 to 4 hr. The culture was then allowed to undergo autolysis by removing it from the shaker and incubating it overnight at 37 C. The lysate was treated with deoxyribonuclease (1 μg/ml) and centrifuged at 6,000 $\times$ *g* for 10 min. The supernatant fluid was sterilized by passage through a 0.45-μm sterile membrane filter.

Recipient strains were streaked on TBAB plates and grown overnight; they were then heavily inoculated into Penassay Broth and grown for 5 hr until maximal motility was obtained. The recipient culture and transducing lysate were mixed in equal volumes and diluted 1:2 into fresh Penassay Broth followed by incubation for 20 min with shaking. The infected cells were washed twice in minimal salts by centrifugation, and were plated on selective media in the presence of sterile PBS1 antiserum. Recombinant clones were picked and restreaked on selective agar. The observed recombination frequency was approximately 10^{-5}/bacterium. Nutritional and phage (Φ25)-resistance markers (*gtaA*) were scored by replica-plating by use of pads of velveteen; flagella markers were scored by replica-plating by use of an inverted flower holder (frog) which was placed onto soft motility agar plates with or without flagella antiserum and incubated at 37 or 46 C, or at both temperatures. Figure 2 shows how flagella markers were identified.

Transformation. Transforming deoxyribonucleic acid (DNA) was isolated by the procedure of Massie and Zimm (9) with the use of lysozyme and Pronase. Transformation was carried out according to the method of Anagnostopoulos and Spizizen (2). Flagella antigenic types were selected by inoculating transformed populations into motility tubes containing antisera specific for the recipient flagella and picking a recombinant that passed rapidly through the tube.

RESULTS

Linkage of hag locus to hisA1. The general location of the *hag* gene on the *B. subtilis* chromosome was established by the use of strains which have antigenically non-cross-reacting flagella (*hag-1* and *hag-2*). A large number of auxotrophic strains were used, all of which required indole (*trp-2*) and one other marker. The second marker was chosen so that linkage with various parts of the chromosome could be demonstrated (Table 2). Transducing lysates were grown on strains with *hag-2* flagella and were used to infect the *hag-1* auxotrophs. Linkage of the *hag* locus was demonstrated (i) by growing recombinants in selective media and measuring *hag-2* flagellar antigen with radio-

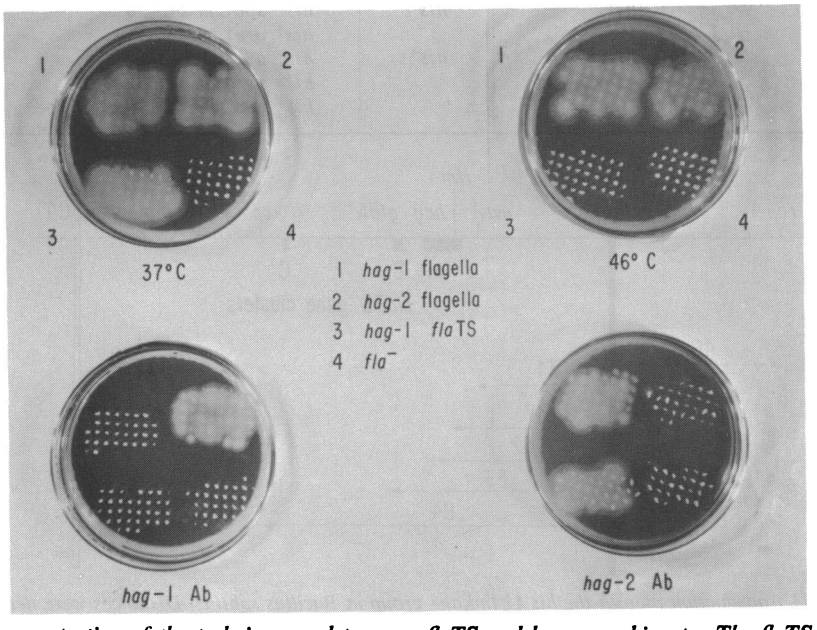

FIG. 2. *Demonstration of the technique used to score flaTS and hag recombinants. The flaTS recombinants were scored by incubation of plates at 37 and 46 C (upper petri plates), and the hag recombinants were identified by use of agar containing flagellar specific antibodies (lower petri plates).*

active antibodies, and (ii) by inoculating the transduced population into motility tubes containing *hag-1* flagellar antibody but lacking a specific nutrient. Under these conditions, only *hag-2* prototrophic recombinants could migrate through the agar. The *hag* locus was found to be linked only to the *cysB* and *hisA1* markers (Table 2). Linkage was observed when the phage were grown on derivatives of 168 strains carrying the *hag-2* locus. When the phage were prepared directly on the W23 strain and used to infect 168 derivatives, no linkage was observed.

Relative map position of the hag locus. The *hisA1* locus has been shown to be linked to a number of mutations. Dubnau et al. (4) mapped *hisA1* between *cysB* and *argC4*, and found 20% linkage to each of these markers. Other markers that have been placed in this region of the chromosome are the phage-resistance markers *gtaA*, *B*, and *C* (12); a sporulation marker, *spoC1*

TABLE 3. *Linkage relationships of markers to hisA1*

Recipient	Donor lysate	Selected marker	Recombinant classes	Cotransduction
				%
BR19	BR13 (*hag-2*)	*his*$^+$	*his*$^+$ *hag-2*, 350/686	51
SB3	BR13 (*hag-2*)	*his*$^+$	*his*$^+$ *hag-2*, 128/260	49
			his$^+$ *cysB*$^+$, 112/564	20
			his$^+$ *cysB*$^+$ *hag-2*, 14/260	5.4
BR85	BR13 (*hag-2*)	*arg*$^+$	*arg*$^+$ *his*$^+$, 0/120	—
BD71	BR13 (*hag-2*)	*his*$^+$	*his*$^+$ *hag-2*, 96/184	52
			his$^+$ *arg*$^+$, 0/120	—
		arg$^+$	*arg*$^+$ *his*$^+$, 0/120	—
BD71	FY'A'	*his*$^+$	*his*$^+$ *rou-1*, 403/673	60
			his$^+$ *rou-1 gtaA*, 236/673	35
			his$^+$ *rou*$^+$ *gtaA*, 340/673	50.5
BR-19 (*hag-2*)	FY'A'	*his*$^+$	*his*$^+$ *hag-1*, 61/90	68
			his$^+$ *hag-1 gtaA*, 48/90	53.5
			his$^+$ *hag-2 gtaA*, 2/90	2
Rog 3	BR13 (*hag-2*)	*his*$^+$	*his*$^+$ *spoC1 hag-2*, 96/192	50
			his$^+$ *spo*$^+$ *hag-2*, 28/192	14.5
			his$^+$ *spo*$^+$ *hag-1*, 8/192	4
BD71	Rog-1	*his*$^+$	*his*$^+$ *spoC1*, 24/150	16
			his$^+$ *arg*$^+$, 0/150	—
IH1057	G-10	*his*$^+$	*his*$^+$ *uvr*$^+$, 108/140	77
			his$^+$ *uvr*$^+$ *hag-2*, 81/140	58
			his$^+$ *uvr*$^+$ *hag-2 gtaA*, 65/140	46.5

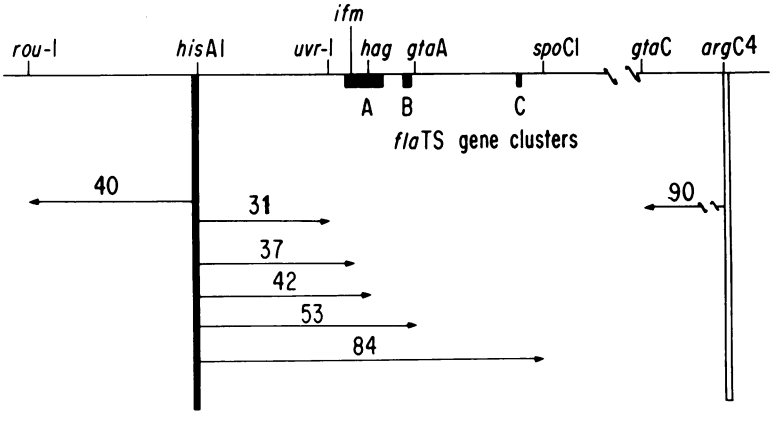

FIG. 3. *PBS1 transduction map of the hisA1 linkage group in Bacillus subtilis. Distances were determined from the average of all experiments carried out during the course of this work (Table 7). Although the relative order of ifm and hag have been established (Table 4), the relative position of the flaTS markers that fall in this region has not as yet been completely determined.*

(10); a locus controlling radiation resistance, *uvr-1*; and a morphological marker, *rou-1*. The results of crosses designed to establish the relative map position of these markers with respect to *hisA1* and *hag* are shown in Table 3 and are summarized in Fig. 3 and Table 7.

Cotransduction of flagella-related markers. Mutations in the *ifm* locus change the relative quantity of flagella per cell as well as the motility of the cells (*see* Materials and Methods); the function that is affected in these mutants is not known, and crosses were designed to determine (i) whether *ifm* clearly segregates from *hag* and (ii) its relative position on the map.

In the first cross, a *hisA1*, *uvr-1*, *hag-2*, *ifm-1* strain was used as recipient, and a *hag-1*, *ifm*$^+$ strain was used as donor. When recombinants were replicated onto 0.4% agar, the *ifm* recombinants could be clearly distinguished (Fig. 1). The data show that *ifm* segregates from *hag-1*;

TABLE 4. *Segregation of ifm, gta, and hag among his*$^+$ *transductants*[a]

(1) Donor MH1, genotype Recipient G25	+ *hisA1*	+ *uvr-1*	+ *ifm-1*	*hag-1* *hag-2*	+ *gtaA*	
	his	*uvr*	*ifm*	*hag*	*gta*	No. in class
	1	0	0	0	0	44
	1	1	0	0	0	13
	1	1	1	0	0	10
	1	1	1	1	0	25
	1	1	1	1	1	48
	1	0	0	0	1	2
No. with donor allele	142	96	83	73	50	142

(2) Donor SC4 genotype Recipient G31	+ *hisA1*	+ *uvr-1*	+ *ifm-1*	*hag-3* *hag-1*	+ *gtaA*	
	his	*uvr*	*ifm*	*hag*	*gta*	No. in class
	1	0	0	0	0	58
	1	1	0	0	0	3
	1	1	1	0	0	8
	1	1	ND[b]	1	0	16
	1	1	ND[b]	1	1	50
	1	0	0	0	1	1
	1	0	ND[b]	1	1	2
	1	1	0	0	1	1
	1	1	1	0	1	1
No. with donor allele	140	79	9[b]	68	55	140

(3) Donor FY'A' genotype Recipient G26	+ *hisA1*	+ *uvr-1*	+ *ifm-1*	*hag-1* *hag-2*	*gtaA* +	
	his	*uvr*	*ifm*	*hag*	*gtaA*	No. in class
	1	0	0	0	0	38
	1	1	0	0	0	8
	1	1	1	0	0	6
	1	1	1	1	0	16
	1	1	1	1	1	68
	1	0	0	0	1	1
	1	1	0	0	1	2
	1	1	1	0	1	2
No. with donor allele	141	102	92	84	73	141

[a] The frequencies of all observed recombinant classes are shown. The donor allele is represented by a 1 and the recipient by a 0.

[b] The *ifm* character of the recombinants that were *hag-3* was not determined. The total therefore represents the recombinants that were *ifm*$^+$, *hag-1*.

of the *his*$^+$ recombinants tested, 10 of 140 were *ifm*$^+$, *hag-2* (Table 4). In a second cross, the donor strain was *hag-3*, *ifm*$^+$, and the recipient was *hisA1*, *uvr-1*, *hag-1*, *gtaA*, and *ifm-1*. Again, *ifm* was found to segregate from *hag*, and 9 of 140 recombinants were *hag-1*, *ifm*$^+$. In the third cross, the same pattern of segregation was found. These data are consistent with the order *uvr-ifm-hag-gta*.

Table 5 shows the results of crosses performed to determine the relationship of the *flaTS* markers to the *hag* locus. In all, 26 temperature-sensitive mutants were tested and approximately 150 *his*$^+$ recombinants were picked in each cross. Since each cross required lysates grown on the *flaTS* strains, a fair amount of variation in the frequency of recombination for the *hag* region was expected. Table 5 includes the extremes in the variation obtained. In spite of these differences, the mutants clearly fall into three distinct groups. Group A includes 21 mutants, all of which map near *hag*. The ratio of the frequency of recombinants for *flaTS* to that for *hag-1* is in the range of 1.1 to 0.92, and the ratio of *flaTS* to *gtaA* is 1.6 to 1.2. Group B contains three mutants; the ratio of *flaTS* to *hag-1* is 0.86 to 0.77, and the ratio of *flaTS* to *gtaA* is 1.0. There is thus far only one mutant that maps in Group C. It is clearly separable from the *hag* and the *gtaA* loci.

To check further the position of the group B mutants, *flaTS-1* and *flaTS-7* were put into a strain carrying the *gtaA* marker and then crossed into BD-71. In this cross, *flaTS-1* was found to be closely linked to *gtaA*; the ratio of recombinants for *flaTS* to those for *gtaA* was 1.05, and the ratio for *flaTS-7* to *gtaA* was 1.2.

TABLE 5. *Transduction of flaTS mutants*

flaTS donor lysate	Percentage of *hisA1* recombinants[a]					Ratio	
	hag-1	*flaTS*	*gtaA*$^+$	*hag-1*, *fla*$^+$	*hag-2*, *flaTS*	*flaTS* to *hag*	*flaTS* to *gtaA*
9	72	80	55	0	5	1.11	1.45
40	68	75	57	1	9	1.10	1.32
4	58	56	40	8	6	0.97	1.40
32	65	62	51	6	4	0.96	1.28
8	66	66	52	6	6	1.00	1.26
22	70	65	42	6	0	0.93	1.55
10	65	66	40	6	7	1.02	1.62
37	74	68	48	6	1	0.92	1.54
7	61	62	42	4	5	1.02	1.48
20	68	72	50	4	8	1.06	1.45
46	55	58	47	2	6	1.05	1.25
1	65	56	57	17	0	0.86	0.99
3	70	54	55	19	7	0.77	0.99
51	58	22	44	39	2	0.38	0.50

[a] The recipient in all these experiments was G-5 (*hisA1*, *hag-2*, *gtaA*). The donor lysates were prepared on the appropriate *flaTS* strain. *hisA1*$^+$ recombinants were picked and tested for the other markers. The results are presented as the percentage of *hisA1*$^+$ recombinants that carry a given marker.

Table 5 also shows that in all the crosses recombinants were obtained that were either *hag-1*, *fla*$^+$ or *hag-2*, *flaTS*, suggesting that all the *flaTS* markers mapped can segregate from the *hag* locus. However, the data obtained thus far do not allow us to assign the precise positions of all of the *flaTS* markers relative to one another and to the *hag* locus.

We have assumed thus far that there is no phase variation in *B. subtilis*. Our results could be complicated if these strains carried two separable *hag* genes and only one was phenotypically expressed. To test this possibility, *hag-3* mutants were used. The *hag-3* gene is derived from *hag-1* by mutation and has been shown to differ in only a single peptide (8). Strains carrying *hag-3* are nonmotile and have flagella that are antigenically identical to the *hag-1* product but lack the normal long-period helix. In the crosses shown in Table 6, recombinants that were nonmotile were picked and tested for antigenic specificity. None of the nonmotile recombinants had *hag-2* antigen, and all of the nonmotile recombinants had flagella. Furthermore, no recombinants of the *hag-1* type were found. In further crosses, over 1,000 recombinants have been picked and tested, and thus far only a single *hag-1* recombinant has been found. Therefore, these data suggest that the strains do not carry cryptic alternate *hag* genes that are linked to *hisA1*.

We have also found that the motility-negative mutation (*mot*) reported by Joys and Frankel (7) does not cotransduce with *hisA1*.

DISCUSSION

Genetic crosses done by use of phage PBS1 established the linkage of the *hag* locus to *hisA1*. The relationship of flagella markers to other markers cotransferred with *hisA1* is shown in Fig. 3 and Table 7.

It was not found possible to demonstrate linkage of *hisA1* to *argC4*, as was reported by Dubnau et al. (4); in fact, the *spoC1* marker of Rogolsky (10) was demonstrated to be less tightly linked to *hisA1* (16%) than the reported level of cotransfer of the *argC4* marker (24%). None of the *hisA1* linked markers could be shown to cotransfer with *argC4*. However, F. E. Young (J. Bacteriol., *in press*) and Grant and Simon (*unpublished data*) have shown that in some specific strains *hisA1* and *argC4* may be

TABLE 6. *Test for a cryptic hag gene*

Recipient	Donor	Motility phenotype	Recombinant classes
G5	SC-3	−	*his*$^+$, *hag-3*, 94/192
hisA1, hag-2, gtaA	*hag-3*	−	*his*$^+$, *hag-3, gtaA*, 42/192
		+	*his*$^+$, *hag-2*, 56/192
G5	SC-4		
hisA1, hag-2, gtaA	*hag-3*	−	*his*$^+$, *hag-3*, 97/200
		−	*his*$^+$, *hag-3, gtaA*, 35/200
		+	*his*$^+$, *hag-2*, 68/200
G22	G10		
hisA1, hag-3	*hag-2, gtaA*	+	*his*$^+$, *hag-2, gtaA*, 79/140
		+	*his*$^+$, *hag-2*, 11/140
		−	*his*$^+$, *hag-3*, 50/140

TABLE 7. *Summary of cotransduction frequencies of markers with hisA1*[a]

Marker	Total fraction of *his*$^+$ recombinants for linked markers	Percentage of cotransfer (*Y*)	Approximate linkage (100 − *Y*)
rou-1	403/673	60	40
uvr-1	775/1,120	69	31
ifm	444/700	63	37
hag	899/1,540	58	42
gtaA	863/1,820	47	53
spoC1	65/412	16	84

[a] The relative order of these markers has been established and was consistently found in all of the transduction experiments. The degree of linkage summarizes our data. The *cysB* marker was found to map to the left of *rou-1* (Table 3).

cotransferred by PBS1 transduction. Young has also shown transformation linkage between the phage-resistance markers *gtaA* and *gtaC* which do not normally cotransfer in PBS1 transduction. This behavior suggests the presence of a chromosomal abnormality or an unstable chromosomal element that can be inserted in this region, e.g., a defective lysogenic phage or an unstable episome. However, more experimentation is obviously necessary to clarify this problem.

All of the *hag* and *flaTS* mutants that we have tested thus far are linked to *hisA1* by cotransduction. Although the data do not allow us to establish unequivocally the position of all of these markers, it is clear that most of them cluster around the *hag* locus. Some mutants (*flaTS-1* and *-3*) appear to be more closely associated with the *gta* locus and segregate from *hag*. One mutant (*flaTS-51*) is clearly separated from the others.

The lack of more markers in this region and the absence of a reliable complementation system in *B. subtilis* has prevented us from establishing discrete functional classes for the *flaTS* mutants. The data available, however, suggest that most of them do not directly affect the structural gene for flagellin. This is certainly clear for the group B and C mutants, which can be readily separated from the *hag* locus. However, even the group A mutants, in almost all of the crosses, were found to segregate from *hag* and give *hag-2*, *flaTS* or *hag-1*, *fla*$^+$ recombinants.

Furthermore, tests of the flagellin proteins of these mutants also indicate that they do not differ from the wild-type protein (Dimmitt and Simon, *unpublished data*). These data suggest that the *flaTS* mutants are defective in ancillary functions that are required for the formation of bacterial flagella. Further work is being directed toward elucidating these functions, and toward determining the gene order in the group A region.

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